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Putative zeatin *O*-glucosyltransferase OscZOG1 regulates root and shoot development and formation of agronomic traits in rice

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Abstract As a ubiquitous reaction, glucosylation controls the bioactivity of cytokinins in plant growth and development. Here we show that genetic manipulation of zeatin-*O*-glucosylation regulates the formation of important agronomic traits in rice by manipulating the expression of *OscZOG1* gene, encoding a putative zeatin *O*-glucosyltransferase. We found that *OscZOG1* was preferentially expressed in shoot and root meristematic tissues and nascent organs. The growth of lateral roots was stimulated in the overexpression lines, but inhibited in RNA interference lines. In shoots, knockdown of *OscZOG1* expression by RNA interference significantly improved tillering, panicle branching, grain number per panicle and seed size, which are important agronomic traits for grain yield. In contrast, constitutive expression of *OscZOG1* leads to negative effects on the formation of the grain-yielding traits with a marked increase in the accumulation levels of *cis*-zeatin *O*-glucoside (cZOG) in the transgenic rice plants. In this study, our findings demonstrate the feasibility of improving the critical yield-determinant agronomic traits, including tiller number, panicle branches, total grain number per panicle and grain weight by downregulating the expression level of *OscZOG1*. Our results suggest that modulating the levels of cytokinin glucosylation can function as a fine-tuning switch in regulating the formation of agronomic traits in rice.

Keywords: *Cis*-zeatin *O*-glucoside (cZOG); cytokinin glucosylation; grain-yield traits; lateral root; panicle development; rice
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INTRODUCTION

Among phytohormones, cytokinins promote cell division and differentiation in plant growth and development from seed germination to senescence (Werner and Schmuelling 2009; Argueso et al. 2010). It is generally accepted that hormonal homeostasis is essential for all physiological and developmental processes in higher plants. The reversible conjugation of phytohormones is suggested to be a mechanism to regulate the pool of the physiologically active forms (Weyers and Paterson 2001; Bajguz and Piotrowska 2009). The naturally occurring cytokinins are adenine derivatives that are classified into isoprenoid or aromatic cytokinins dependent on the nature of the N^6-side chain (Mok and Mok 2001). There are four members in the group of isoprenoid cytokinins, including N^6-($\triangle^2$-isopentenyl) adenine (iP), *trans*-zeatin (tZ), dihydrozeatin, and *cis*-zeatin (cZ). Among isoprenoid cytokinins, *trans*-zeatin is considered to play a central role in plant growth and development due to its general occurrence and extremely high activity in most bioassays (Mok and Mok 2001).

Cytokinins can be glucosylated to form *O*-glucosides and N-glucosides, generally assumed to be storage products of cytokinins and all of these cytokinin glycosides are known to be inactive (Martin et al. 1999b; Mok et al. 2000a; Mok and Mok 2001). *O*-glucosylation conjugation of cytokinins is considered to be reversible and the *O*-glucosides are resistant to cleavage of the N^6-side chain by cytokinin oxidases (Jones and Schreiber 1997; Mok and Mok 2001; Schmulling et al. 2003). An enzyme with β-glucosidase activity, converting zeatin *O*-glucoside to zeatin, was identified in maize and the corresponding gene (*Zm-p60.1*) was highly expressed in root meristem (Brzobohaty et al. 1993). Glycosyl moiety can be transferred from an activated glycosyl donor to hydroxyl group in the side chain of cytokinin by specific glycosyltransferase enzymes. Enzymes and genes involved in zeatin glycosylation have been extensively studied. The first zeatin *O*-glucosyltransferase was isolated from immature *P. lunatus* seeds (Dixon et al.

Abbreviations

cZ	*cis*-zeatin
cZOG	*cis*-zeatin O-glucoside
IM	inflorescence meristems
iP	N^6-(Δ^2-isopentenyl) adenine
LC-MS	liquid chromatography-mass spectrometry
LC-QTOF-MS	liquid chromatography quadrupole time-of-flight mass spectrometry
LRI	lateral root initiation
LRP	lateral root primordial
qRT-PCR	quantitative PCR with reverse transcription
SAM	shoot apical meristem
tZ	*trans*-zeatin
tZOG	*trans*-zeatin *O*-glucosides

1989). Subsequently, the zeatin *O*-glucosyltransferase (ZOG1) and *O*-xylosyltransferase (ZOX1) from *Phaseolus* (Martin et al. 1999b, 1999a) as well as two cytokinin *N*-glucosyltransferase genes in *Arabidopsis* were identified (Hou et al. 2004). As the common modification of adenine ring of cytokinins such as *trans*-zeatin, dihydrozeatin and N^6-($\triangle^2$-isopentenyl) adenine, *N*-glucosylation can occur at the N^7- and N^9-position (Mok and Mok 2001). Although both *O*-glucosylation and *N*-glucosylation are common modifications of cytokinins, a lot of studies have been concentrated on *O*-glucosylation because of the availability of the specific genes encoding *O*-glucosyltransferase enzymes (Mok and Mok 2001).

Cytokinin plays a central role in regulating the activity of the reproductive shoot apical meristem (SAM) (Veit 2009; Werner and Schmuelling 2009; Argueso et al. 2010), which is one parameter determining seed yield in crop plants. The reduced expression of *OsCKX2* causes cytokinin accumulation in inflorescence meristems (IM) and increases the number of reproductive organs, resulting in enhanced grain yield (Ashikari et al. 2005). Therefore, suitable architecture of crop plants for high grain yield can be achieved by genetic manipulation of the bioactive cytokinin level. In previous studies, transformants and mutants of *Arabidopsis* with either reduced cytokinin levels or deficient in cytokinin perception exhibit slower growth rates and reduced plant stature (Werner et al. 2003; Higuchi et al. 2004; Nishimura et al. 2004; Riefler et al. 2006). In this study, we generated transgenic rice lines by interfering or overexpressing *OscZOG1* gene, encoding a putative zeatin-*O*-glucosyltransferase in order to investigate whether genetic manipulation of cytokinin bioactivities is capable of promoting formation of agronomic traits in rice. Our findings demonstrate the feasibility of improving the critical yield-determinant agronomic traits, including tiller number, panicle branches, total grain number per panicle and grain weight by downregulating the expression level of *OscZOG1*.

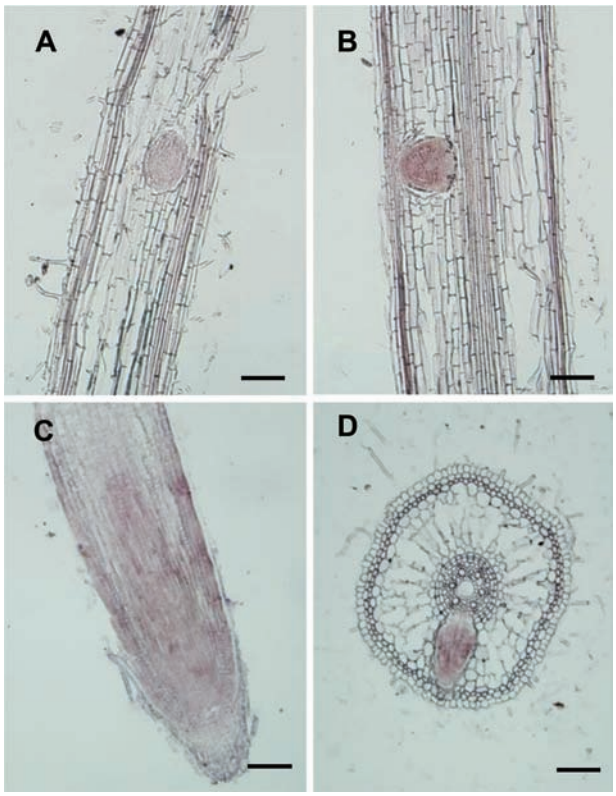

Figure 1. *OscZOG1* is highly expressed in primary root meristem and lateral root primordia in rice
(**A**–**D**) In situ hybridization analysis of *OscZOG1* in roots of 4-d-old rice seedlings. The sections of lateral root primordia (**B**, **D**) and primary root tip (**C**) of wild-type roots were hybridized using anti-sense (**B**, **C** and **D**) or sense (**A**) *OscZOG1* probe. Scale bar = 100 μm.

RESULTS

OscZOG1 is preferentially expressed in root meristem and lateral root primordia

During our initial studies, we were particularly interested in identifying novel genes and pathways that contributed to the regulatory networks involved in the lateral root development in rice. We found that *OscZOG1*, encoding a putative zeatin-*O*-glucosyltransferase, was highly expressed in root meristematic regions of 4-d-old rice seedlings by performing in situ hybridization experiments (Figure 1A–D). The sections of lateral root primordia and primary root tip of the wild-type roots were hybridized using anti-sense or sense *OscZOG1* probes. In situ hybridization data showed that in roots of 4-d-old rice seedlings, *OscZOG1* expression concentrated in the tips of primary roots (Figure 1C) and lateral root primordia (Figure 1B, D). Strong hybridization signals were found throughout the primary root tip, including the root meristematic region and the signal intensity began to dramatically decrease over the tip region (Figure 1C). It is well known that lateral root initiation begins with the activation of cell proliferation in pericycle cells, which produces a lateral root primordium (Laskowski et al. 1995). Next, *OscZOG1* expression was examined during the initiation of lateral roots. Strong signals were observed in primordia, which formed a new meristem during the initiation of lateral roots in rice (Figure 1B, D). Almost no expression was detected in the epidermis and internal layers of the root. These results indicate that *OscZOG1* is activated preferentially in dividing regions in roots.

OscZOG1 regulates lateral root development

In the experiments described above, we examined the expression patterns of *OscZOG1* during lateral root initiation. To uncover how *OscZOG1* modulates lateral root development, we generated the RNAi and overexpression transgenic lines of *OscZOG1*, respectively and the expression levels of *OscZOG1* in these transgenic lines compared with wild type were verified using quantitative PCR with reverse transcription (qRT-PCR) (Figure 2A, B). We examined growth of lateral roots for the transgenic lines propagated under liquid culture conditions in greenhouse or in the field. Under both growth conditions, initiation and growth of the lateral roots significantly increased in the overexpression lines, but were reduced in the RNAi lines compared with the wild

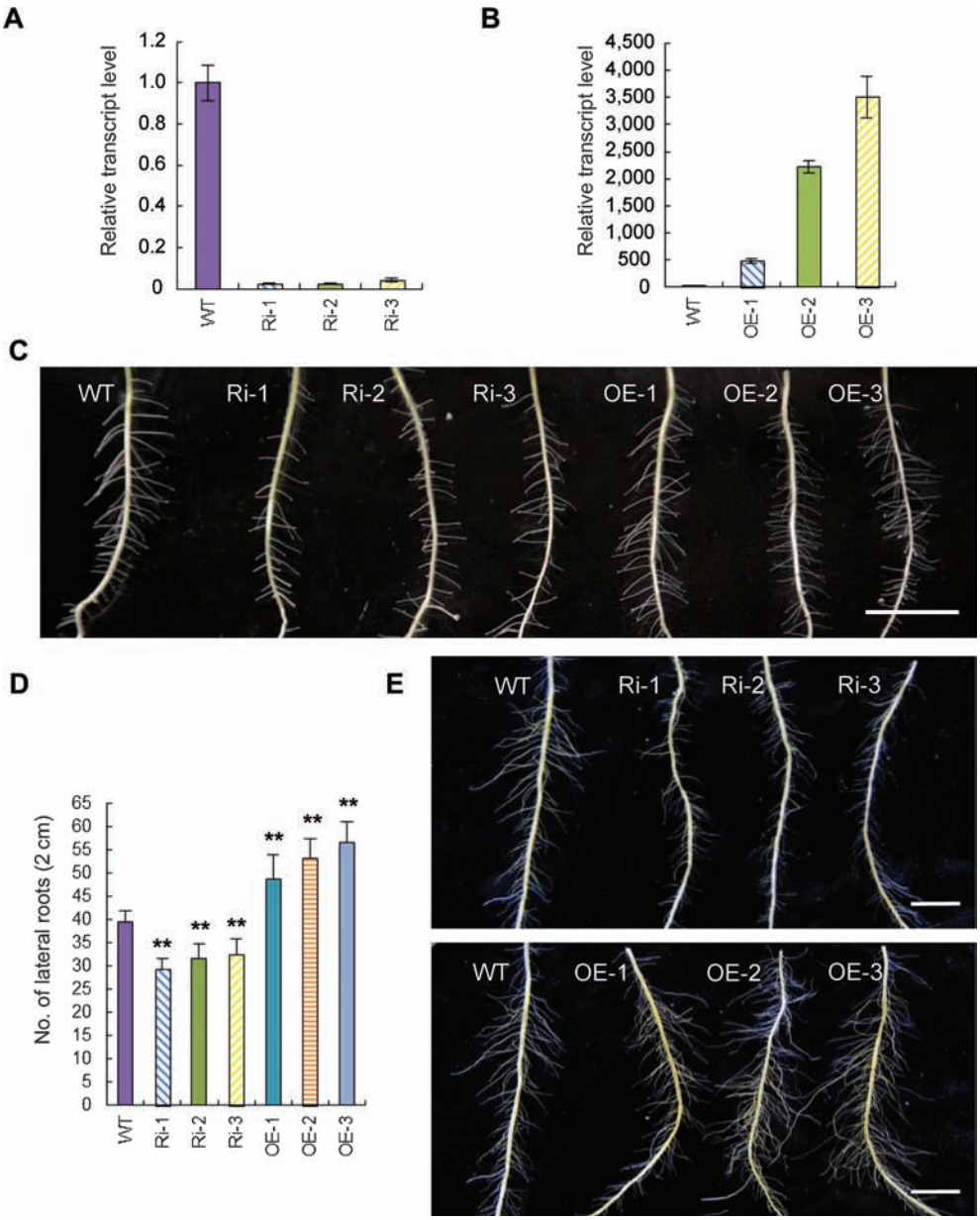

Figure 2. Modulation of *OscZOG1* expression affects lateral root development in rice

(**A**, **B**) qRT-PCR analysis of *OscZOG1* mRNA abundance in three RNAi (Ri-1, Ri-2 and Ri-3) (**A**) and three overexpression (OE-1, OE-2 and OE-3) (**B**) transgenic lines of rice, respectively. *OsACTIN* (LOC_Os03g50885) was used as the internal standard. Error bars indicate standard deviations of three technical replicates, and the results were consistent in three biological replicates. (**C**) Phenotypes of lateral root growth in the 2-week-old seedlings of WT and the transgenic lines. Scale bar = 1 cm. (**D**) Statistical analyses of number of lateral roots per plant in WT and the transgenic lines ($n = 10$). Lateral root numbers continually counted in 2-cm length along the primary root from shoot-root junction regions. Statistical analyses were performed ($^{**}P$ value < 0.01, Student's *t* test). Error bars indicate *SD*. (**E**) Phenotypes of lateral root growth in roots of the 4-week-old seedlings of WT and the transgenic lines grown in field. Scale bar = 1 cm.

type (Figure 2C–E). These results show that *OscZOG1* function is evident during initiation and growth of lateral roots in rice.

***OscZOG1* functions in shoot-borne crown root development**

As one of the major root types in rice, the shoot-borne crown roots are initiated at the lower stem nodes (Hochholdinger et al. 2004). In situ hybridization data revealed that *OscZOG1* was activated in crown root initials (Figure 3B), and was highly expressed in growing crown primordia (Figure 3C–F). Therefore, the activation of *OscZOG1* in crown root formation proceeded in a similar manner as observed in the lateral root initiation (Figure 1B, D). The growth and development of crown roots was

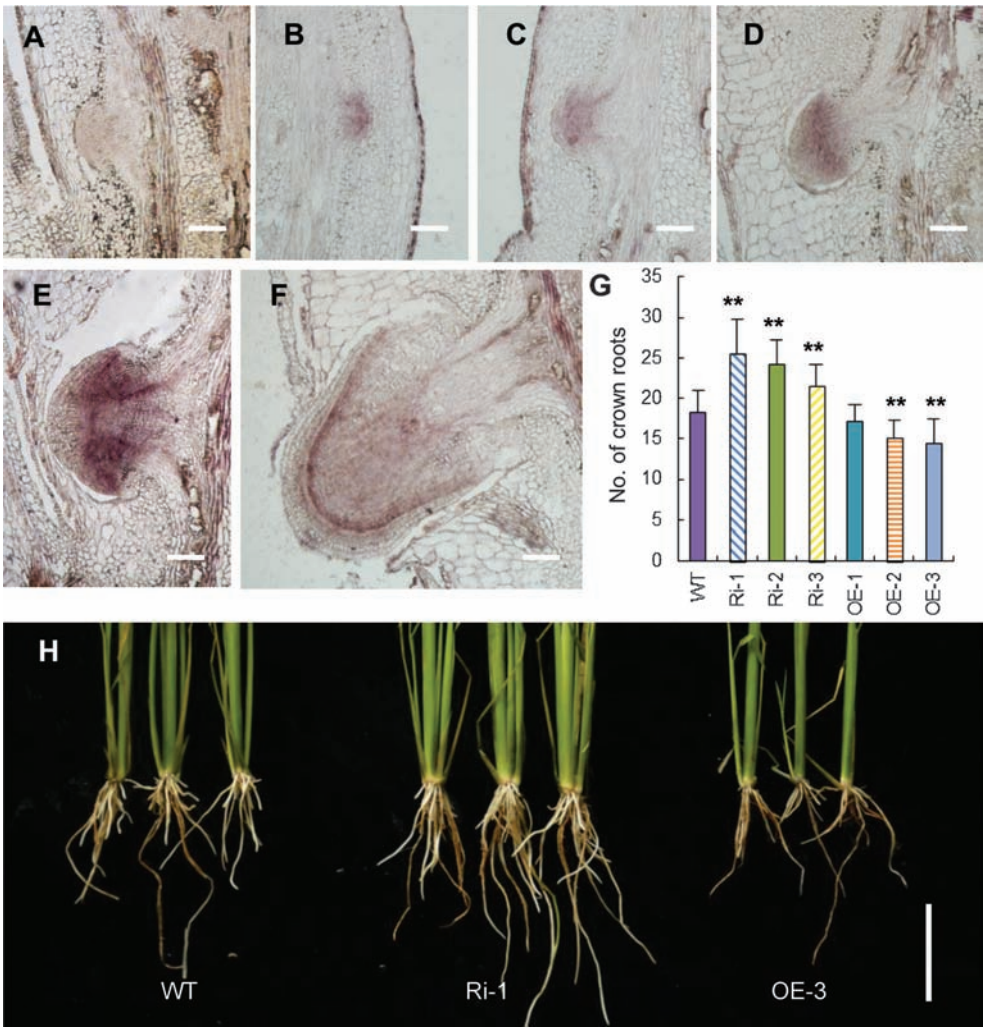

Figure 3. Modulation of *OscZOG1* expression regulates crown root development in rice
(**A**–**F**) In situ hybridization in the sections of crown root primordia at different developmental stages. The sections of wild type seedlings were hybridized using anti-sense (**B**–**F**) or sense (**A**) *OscZOG1* probe. Scale bar = 100 μm. (**G**) Average number of crown roots per plant of WT, the RNAi and overexpression transgenic lines grown in field for 4 weeks (n = 20). Statistical analyses were performed (**P value < 0.01, Student's t test). Error bars indicate SD. (**H**) Phenotypes of crown roots in 4-week-old plants of WT, the RNAi and overexpression transgenic lines grown in field. Scale bar = 5 cm.

examined for the rice transgenic lines grown in the field for 4 weeks. Under field growth conditions, initiation and growth of the crown roots significantly increased in the RNAi lines, but were reduced in general in the overexpression lines compared with wild-type (Figure 3G, H). These results indicate that *OscZOG1* plays a critical role during initiation and growth of crown roots in rice.

OscZOG1 is highly expressed in shoot meristematic tissues and functions in early seedling growth and tillering

To obtain a better understanding of *OscZOG1* functions in shoot development, we also examined the spatiotemporal patterns of *OscZOG1* mRNA accumulation in both vegetative and reproductive phases of development by performing in situ hybridization experiments. As shown in Figure 4B, the strong signals were found in shoot apical meristem (SAM), leaf primordia and young leaves. Further examination of the *OscZOG1* mRNA accumulation during inflorescence development revealed that all meristematic tissues showed strong signals, including branch meristems (BM) and inflorescence meristems (IM) (Figure 4E–G). Also, the strong signals were observed in axillary buds (Figure 4C, D).

Next, the time course of *OscZOG1* expression was examined during spikelet development. During early developmental stages of spikelets, the strong hybridization signals were observed in floral meristem (FM) and the primordia of glumes, palea and lemma in out whorls (Figure 4H, I). While the stamen primordia were continuing to grow, *OscZOG1* expression concentrated on the remaining tissue of the central meristem and developing stamens (Figure 4J–M). At a late stage of spikelet development, the strong hybridization signals were observed in anther locules and filaments (Figure 4N–P). The presence of

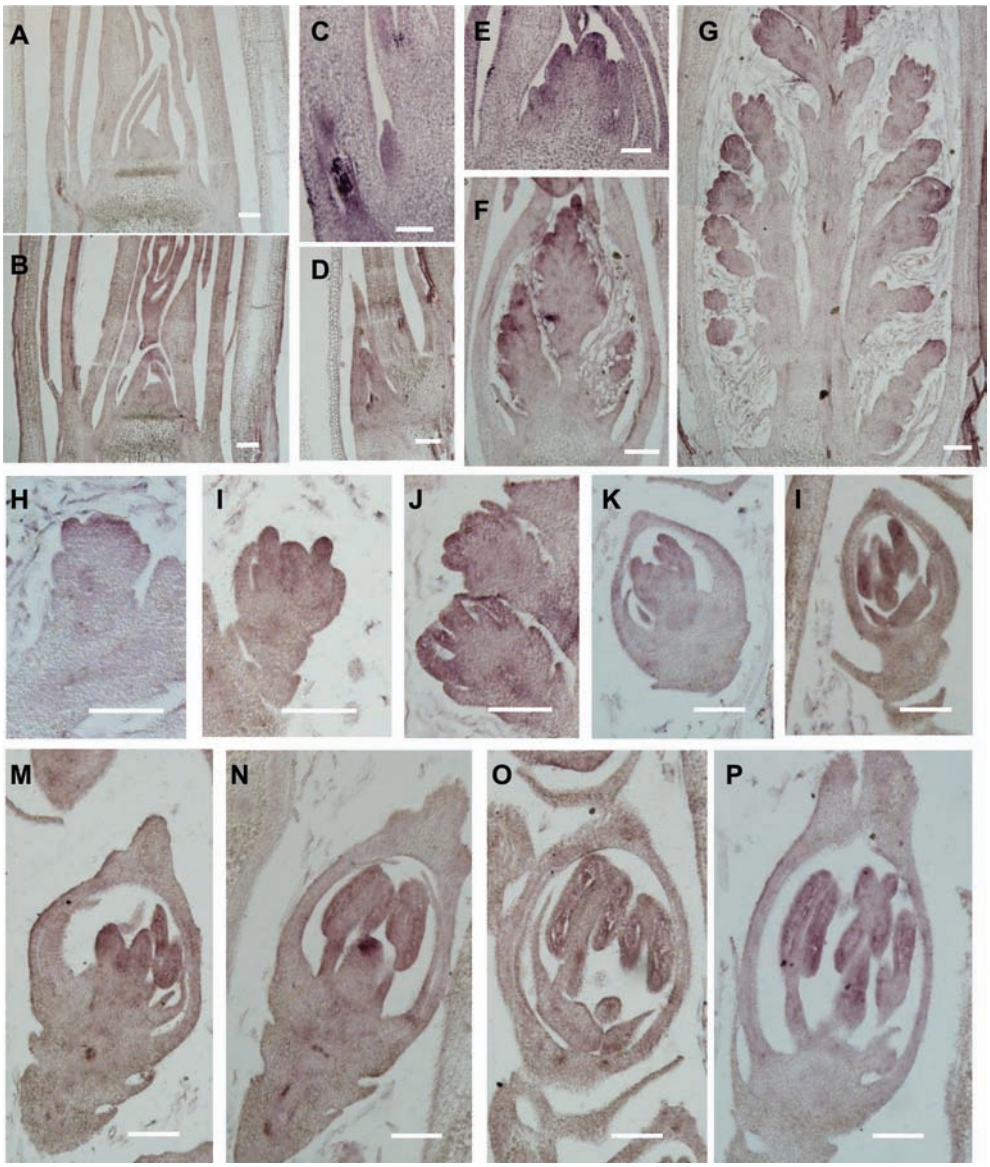

Figure 4. *OscZOG1* is highly expressed in shoot, inflorescence and crown root meristems in rice
In situ hybridization in the sections of shoot apical meristem and young leaves (**A**, **B**), axillary bud (**C**, **D**), branch meristem (**E**, **F**) and spikelet at different development stages (**G**–**P**). The sections of wild type seedlings were hybridized using anti-sense (**B**–**P**) or sense (**A**) *OscZOG1* probe. Scale bar = 100 μm.

strong hybridization signals in the meristematic tissues of inflorescence suggests that *OscZOG1* expression is targeted to cells in rapidly growing regions.

The early activation of *OscZOG1* expression in SAM, leaf primordia and young leaves suggest that *OscZOG1* may play a critical role in early seedling growth. Our first test was to examine SAM size in the seedlings of the wild-type, RNAi and overexpression transgenic lines grown in soil for 1 month in a greenhouse. Observation of a longitudinal section of the shoot apex revealed that the SAM was morphologically normal and exhibited an enlarged size in the RNAi transgenic line Ri-1, but the SAM size in the overexpression line OE-3 was similar to that of the wild-type seedlings (Figure 5A, B). In comparison, the RNAi transgenic lines exhibited a higher plant height, whereas the plant height of the overexpression lines was reduced with respect to the wild-type seedlings when grown in liquid culture conditions in greenhouse for 2 weeks (Figure 6A, B). Strikingly, we also observed a drastic enhancement of tiller numbers in the RNAi transgenic lines, but a significant decrease in the overexpression lines in comparison with the wild-type seedlings (Figure 6C, D). For instance, the tiller development in the RNAi transgenic line Ri-1 was at a faster rate than was the tiller of the wild type (18.3 tillers on average for Ri-1 and 12.3 tillers for wild type) whereas a significant reduction in tiller number was observed for the overexpression line OE-3 (7.3 tillers for OE-3) (Figure 6C, D). These results indicate that *OscZOG1* plays a critical role in vegetative growth

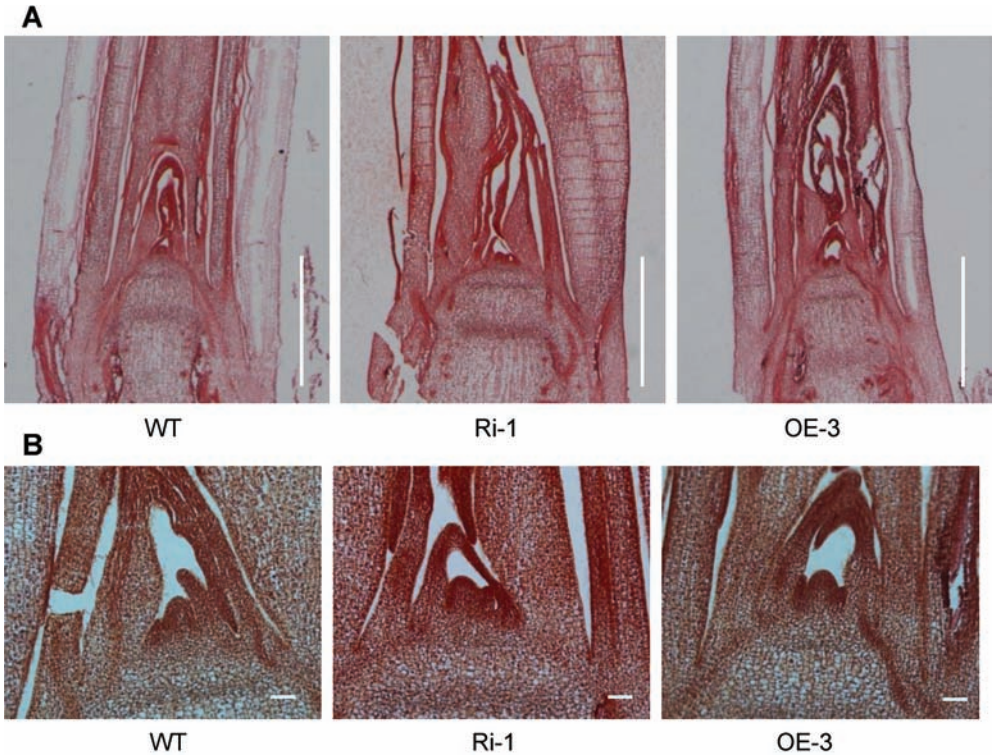

Figure 5. Effects of overexpression or interference with *OscZOG1* expression on SAM maintenance and young leaf development in rice

(**A**) Longitudinal sections of shoot apical meristems of the seedlings of WT, RNAi and overexpression transgenic lines grown in soil for 1 month in greenhouse. Bar = 1 mm. (**B**) Close-up images of longitudinal sections of shoot apical meristems of the genotypes as indicated in (**A**). Bar = 50 μm.

and development, affecting seedling growth and tiller development.

OscZOG1 modulates adult plant height and flag leaf senescence

We measured the heights of 13-week-old plants grown in the field. As shown in Figure 7A, a significant enhancement of plant heights was observed in the RNAi transgenic lines whereas the plant heights were reduced in the overexpression lines compared to the wild-type plants. For instance, plant height was increased by 12.6% in the RNAi transgenic line Ri-1 at the time of grain filling, whereas a 10.1% reduction in plant height was observed for the overexpression line OE-3 (Figure 7A). The heights of the RNAi transgenic lines were still higher than those of the wild-type plants, even at plant maturity (Figure 7B). These results suggest that *OscZOG1* functions as a fine-tuning regulator in modulation of plant heights through the life span.

As a developmentally controlled degenerative process, leaf senescence is induced by exogenous signals such as light and water deficits and regulated by endogenous factors such as ethylene and cytokinin (Gan and Amasino 1995; Buchanan-Wollaston et al. 2003; Guo and Gan 2005; Lim et al. 2007). Rice flag leaves act as the major source of phloem-delivered photoassimilates for developing seeds, and are also believed to be a major source of remobilized metals to seeds (Grusak and DellaPenna 1999; Narayanan et al. 2007). At the developmental stage of plant maturity, a noticeable difference in flag leaf senescence was observed between the wild-type plants and the transformants of the overexpression lines (Figure 7C). The overexpression lines exhibited accelerated flag leaf yellowing phenotypes compared to the wild-type plants, whereas the flag leaves of the RNAi lines showed a similar phenotype relative to those of the wild-type plants under natural senescence conditions (Figures 7C, S1A, B).

OscZOG1 regulates panicle development and seed formation

The architecture of a mature rice panicle, which consists of one rachis (main axis), several primary rachis branches, tens of secondary rachis branches as well as the number of grains setting on these branches, is one of the most important agronomic traits that contribute directly to grain yield (Sakamoto and Matsuoka 2008). Importantly, downregulation of *OscZOG1* expression by RNA interference can generate the rice transgenic plants in which important agronomic traits for grain yield were significantly improved, including increase in panicle branching, grain number per panicle and seed size. In general, the transgenic rice lines overexpressing *OscZOG1* led to reduction in panicle size and the number of branches, especially for secondary branches (Figure 8A–D). By contrast, the RNAi-*OscZOG1* transformants had longer panicles with more branches relative to the wild-type plants (Figure 8A–D). Considering that *OscZOG1* is highly expressed in reproductive

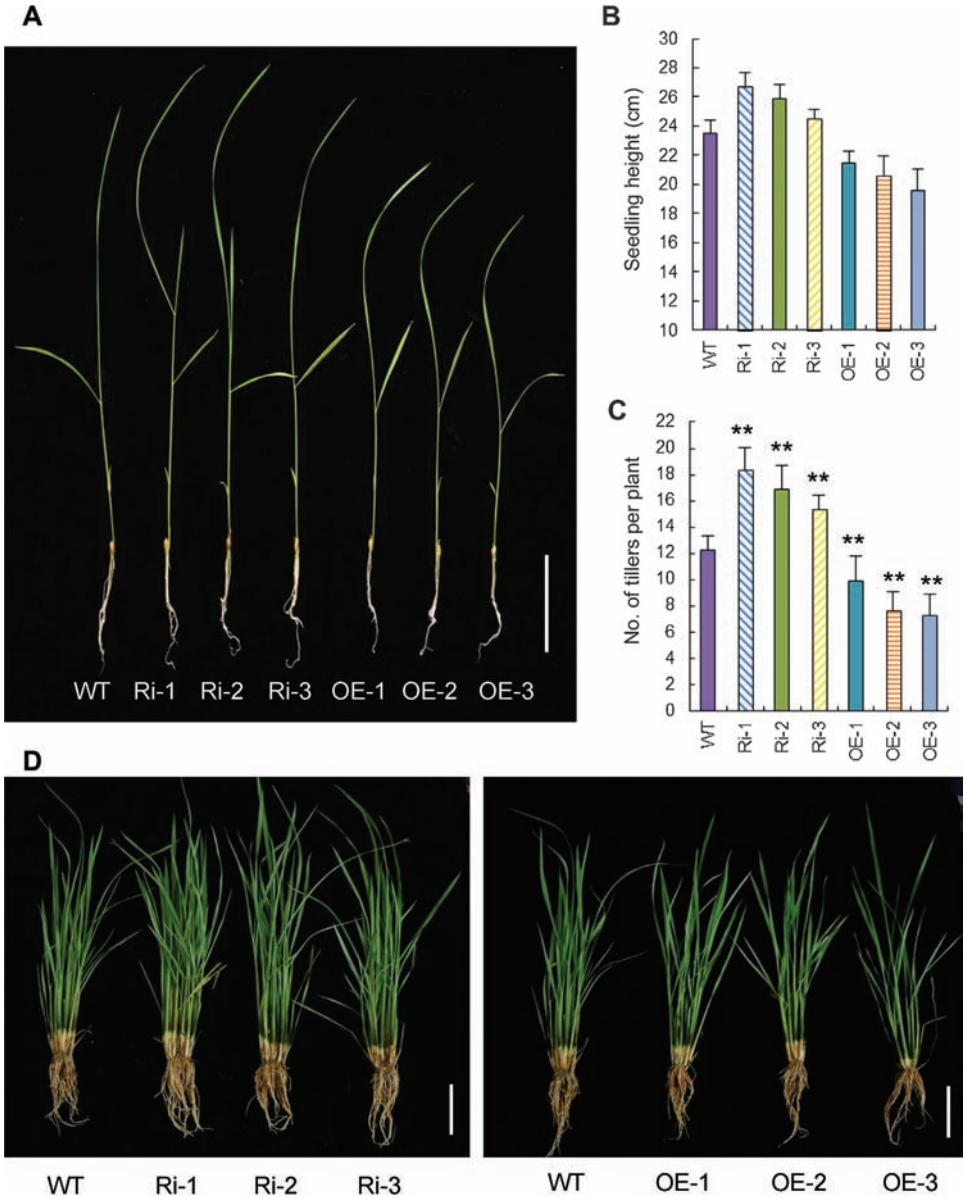

Figure 6. Effects of overexpression or interference with *OscZOG1* expression on plant growth and tiller development in rice
(**A**) Phenotypes of the 2-week-old seedlings of WT, the *OscZOG1*-RNAi and over-expression transgenic lines. Scale bar = 5 cm. (**B**) Seedling height measurements as shown in (**A**) ($n = 10$). Statistical analyses were performed ($^{**}P$ value < 0.01, Student's *t* test). Error bars indicate SD. (**C**) Average number of tillers per plant of each genotypes as shown in (**D**) ($n = 10$). Statistical analyses were performed ($^{**}P$-value < 0.01, Student's *t*-test). Error bars indicate *SD*. (**D**) Phenotypes of the 7-week-old seedlings of WT, the *OscZOG1*-RNAi and over-expression transgenic lines grown in field. Scale bar = 10 cm.

meristematic tissues (Figure 4), the genetic modulation of *OscZOG1* expression also determines the number of grains per panicle that is an important yield trait in rice. The number of grains per panicle dramatically increased to 172–186 on average per panicle in three RNAi transgenic lines with respect to 156 per panicle in the wild-type panicles, whereas three overexpression lines showed a significant reduction in the number of grains per panicle (121–138 per panicle on average) (Figure 8E). The RNAi-*OscZOG1* transformants had more spikelets per panicle, which is attributable to more primary and secondary branches generated in the transformants relative to the wild-type plants. More importantly, downregulating the expression level of *OscZOG1* led to a significant increase in 1000-grain weight ranged from 28.2 to 29.5 g in three RNAi-transgenic lines with respect to 27.6 g in the wild-type grains (Figure 9A, B). By contrast, overexpressing *OscZOG1* negatively modulated grain development (Figure 9A, B). These results of phenotypic analysis indicate that cytokinin glucosylation through genetic modulation of *OscZOG1* represents a fine-tuning of regulating the formation of important agronomic traits, especially for the yield contributing factors such as tiller number,

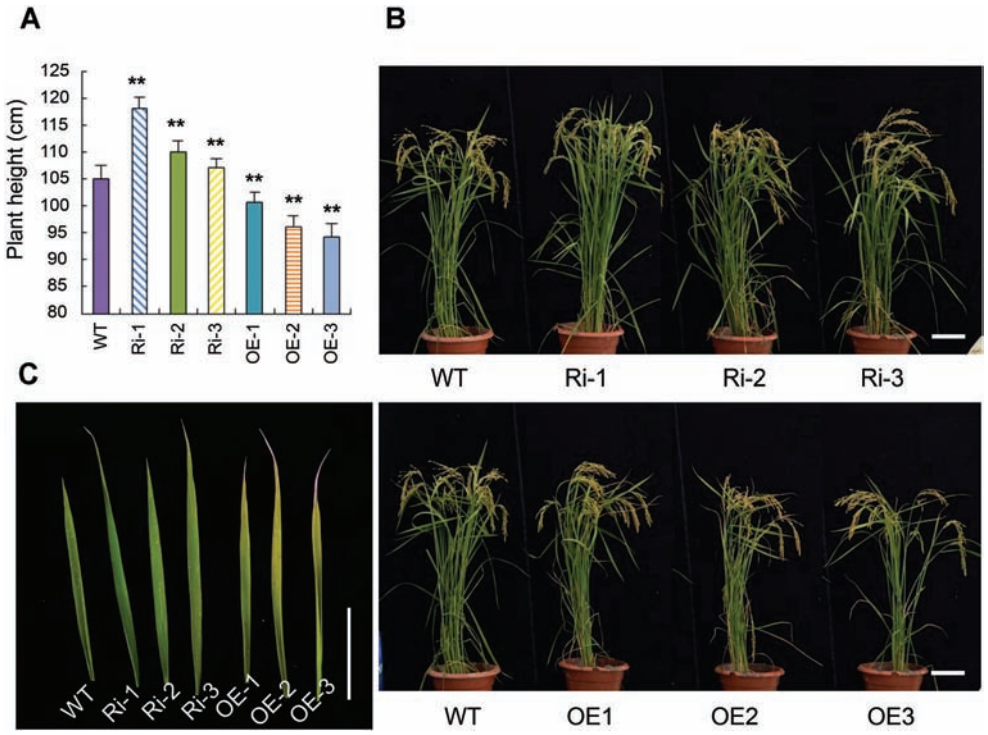

Figure 7. Effects of overexpression or interference with *OscZOG1* expression on plant growth and flag leaf senescence in rice
(**A**) Plant height measurements of the 13-week-old plants of WT, the *OscZOG1*-RNAi and overexpression transgenic lines grown in field ($n = 20$). Statistical analyses were performed ($^{**}P$-value < 0.01, Student's *t*-test). Error bars indicate *SD*. (**B**) Phenotypes of the 14-week-old plants of wild-type, the *OscZOG1*-RNAi and overexpression transgenic lines grown in field. Scale bar = 10 cm. (**C**) Senescence phenotypes of flag leaves detached from the plants of the different genotypes as shown in (**B**). Scale bar = 10 cm.

panicle branches, total spikelet number per panicle and grain weight.

Over-expressing *OscZOG1* leads to accumulation of cZOG in transgenic plants

According to the accumulated reports, *O*-glucosylzeatin is found in all plant species examined (Mok and Mok 2001). As shown in Figure S2, the phylogenetic relationship analysis of OscZOG1 with the reported zeatin-*O*-glucosyltransferases indicate that OscZOG1 shares a sequence similarity with UGT85A1 in *Arabidopsis* (Jin et al. 2013), but much less similarity with ZOG1 in *P. lunatus* (Martin et al. 1999b), *cis*ZOG1 and *cis*ZOG2 in maize (Martin et al. 2001b; Veach et al. 2003), and cZOGT1, cZOGT2 and cZOGT3 in rice (Kudo et al. 2012). Interestingly, subcellular localization analysis showed that OscZOG1 was found to be localized both in cytoplasm and nucleus (Figure S3), which is similar to the localization patterns observed for UGT85A1 (Jin et al. 2013) and the other two *Arabidopsis* UGTs (Husar et al. 2011; Wang et al. 2012). In order to test the impact of modulating the expression of *OscZOG1* on the accumulation of *O*-glucosylzeatin, cytokinins were purified from shoots of the young transgenic rice plants and quantified by liquid chromatography-mass spectrometry (LC-MS). As shown in Figure 10A–D, *O*-glucosides of *cis*-zeatin (cZOG) were found to be increased significantly in shoots of the *OscZOG1*-overexpression transgenic rice line (OE-3) while no significant differences were found in the levels of *trans*-zeatin *O*-glucosides (tZOG) between wild-type and the overexpression transgenic line OE-3 (Figure S4A). In contrast, the amounts of cZOG were lower in shoots of the *OscZOG1*-RNAi transgenic line (Ri-1) than that in the wild-type shoots (Figure 10A). The levels of *trans*-zeatin and *cis*-zeatin were varied slightly among wild-type, the *OscZOG1*- RNAi transgenic line and *OscZOG1*-overexpression transgenic line (Figure S4B and C), which is consistent with the findings reported in *UGT85A1* overexpression transgenic lines in *Arabidopsis* (Jin et al. 2013).

DISCUSSION

The specific mechanisms by which cytokinins regulate crop production and grain development have long been investigated in a variety of plant species such as maize (Lur and Setter 1993; Cheikh and Jones 1994; Rodo et al. 2008), rice (Ashikari et al. 2005), barley (Zalewski et al. 2010), chickpea (Emery et al. 1998). For most of the plant breeders, the priority is maximization of yield that is considerably controlled by cytokinins. Thus, the modulation of cytokinin biosynthesis, degradation and modification can be an intrinsic tool for increasing grain yield.

In this study, we generated the rice transgenic plants in which *OscZOG1* expression was downregulated by RNA interference. Importantly, the agronomic traits for grain yield

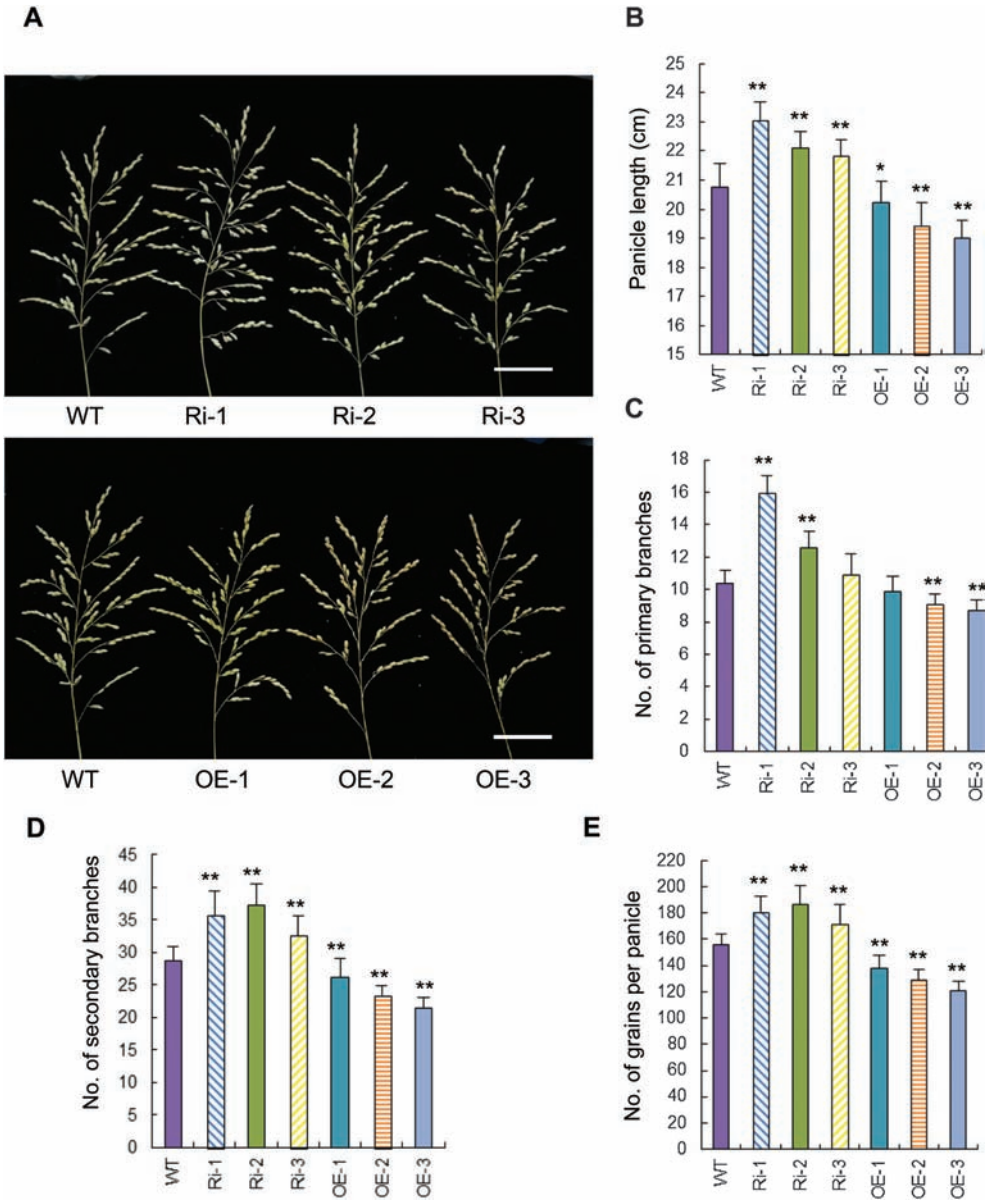

Figure 8. Effects of overexpression or interference with *OscZOG1* expression on panicle development in rice
(**A**) Panicle phenotypes of WT, the *OscZOG1*-RNAi and overexpression transgenic lines grown in field. (Scale bar = 5 cm). (**B**–**E**) Characterization of main panicle parameters, including panicle length (**B**), number of primary branches (**C**), number of secondary branches (**D**) and number of grains per panicle (**E**) in the different genotypes as shown in (**A**) ($n = 20$). Statistical analyses were performed. ($^{*}P$ value < 0.05, $^{**}P < 0.01$. Student's *t*-test). Error bars indicate *SD*.

in these RNAi-*OscZOG1* transformants were significantly improved, including increase in panicle branching, grain number per panicle and seed size. Panicle architecture is one of the essential factors used to determine the yield of cereal crops. By contrast, the transgenic rice lines over-expressing *OscZOG1* led to reduction in panicle size and number of primary and secondary branches. Based on genetic evidence and experimental data, the possibility was raised that cytokinin glycosylation through genetic modulation of the *OscZOG1* expression represents a fine-tuning of regulating the formation of important agronomic traits, especially for the yield contributing factors such as tiller number, panicle branches, total spikelet number per panicle and grain weight. More importantly, our findings demonstrate the feasibility of improving the critical yield-determinant agronomic traits by modulating the expression level of *OscZOG1* in rice breeding practice.

Among hormones, cytokinin plays a central role in regulating the activity of the reproductive SAM (Veit 2009; Werner and Schmuelling 2009), which is one parameter determining seed yield. Cytokinins regulate plant growth and development from seed germination to senescence by promoting cell division and differentiation (Gan and Amasino 1995; Mok and Mok 2001; Guo and Gan 2005;

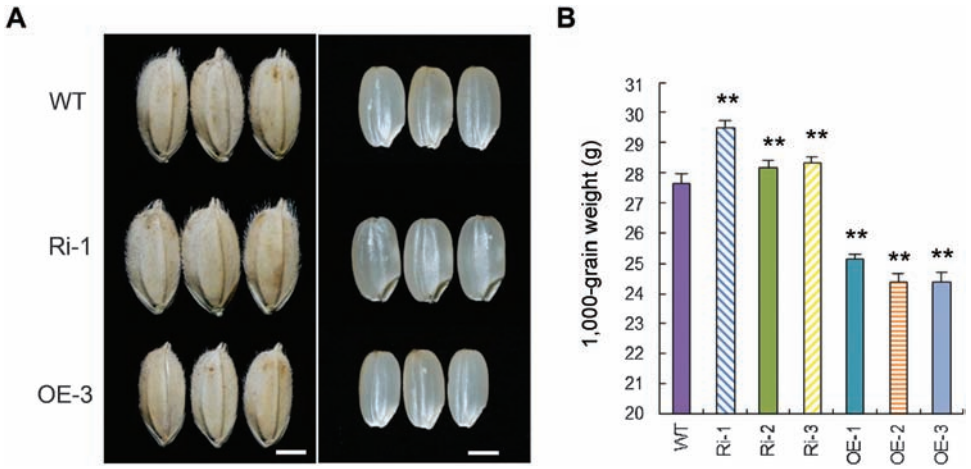

Figure 9. Modulation of *OscZOG1* expression regulates seed formation in rice
(**A**) Seeds of WT, the *OscZOG1*-RNAi and overexpression transgenic lines grown in field. Scale bar = 2 mm. (**B**) Characterization of 1000-grain weights of *OscZOG1* RNAi and overexpression transgenic lines ($n = 10$). Statistical analyses were performed ($^{**}P < 0.01$, Student's *t*-test). Error bars indicate *SD*.

Werner and Schmuelling 2009; Argueso et al. 2010). Several lines of evidence suggest that reduction of the cytokinin status abbreviates the activity of the SAM, indicating that cytokinin is a positive regulator of SAM activity (Werner et al. 2001; Nishimura et al. 2004; Werner and Schmuelling 2009). It is known that zeatin is an essential cytokinin in higher plants due to its ubiquitous nature and high activity. Zeatin and its derivatives are the most important group of isoprenoid cytokinins. Among isoprenoid cytokinins, *trans*-zeatin is considered to play a central role in plant growth and development due to its extremely high activity in most bioassays (Mok and Mok 2001). Other free bases with cytokinin activity, *cis*-zeatin, dihydrozeatin, and N^6-($\triangle^2$-isopentenyl) adenine (iP), are also present in most plant tissues (Mok and Mok 2001; Sakakibara 2006). Interestingly, evidence from recent studies suggests that *cis*-zeatin may act as an active cytokinin in maize (Martin et al. 2001b; Veach et al. 2003) and rice (Kudo et al. 2012). It has been recognized that the relative stability in the levels of other cytokinins is necessary for maintaining cytokinin homeostasis in plant tissues since small decreases in zeatin concentrations could trigger zeatin biosynthesis in organs and tissues capable of cytokinin biosynthesis, such as root tips and shoot meristems (Mok and Mok 2001; Sakakibara 2006; Muller and Sheen 2008; Werner and Schmuelling 2009; Perilli et al. 2010). With regards to the role of *OscZOG1* in improving crop production, it is worth noting that the major contributions of downregulating *OscZOG1* expression lie in promoting meristematic activity in both vegetative and reproductive developmental stages without undesirable phenotypes such as abnormal leaf, flower or grain morphology. Considering that *OscZOG1* is highly expressed in reproductive meristem tissues, genetic manipulation of cytokinin conjugation status, which has been achieved by up- or downregulating the expression level of *OscZOG1*, can moderately promote or inhibit the activity of the inflorescence meristem. In maize transgenic plants, increased zeatin conjugation caused by constitutively expressing *ZOG1*, encoding a zeatin *O*-glucosyltransferase from *Phaseolus lunatus* L., leads to a pronounced reduction in tassel size, branching, and spikelet production (Rodo et al. 2008). Similar results were described in the previous studies that genetic manipulation of cytokinin glycosylation by overexpressing *ZOG* genes can significantly alter the phenotypes of transformants in tobacco (Martin et al. 2001a) and rice (Kudo et al. 2012). In contrast, the reduced expression of *OsCKX2* causes cytokinin accumulation in inflorescence meristems and increases the number of reproductive organs, resulting in enhanced grain yield (Ashikari et al. 2005). Our findings are consistent with previous reports and further confirm that suitable architecture of crop plants for high grain yield can be achieved by genetic manipulation of the bioactive cytokinin level. In recent reports, it has been increasingly recognized that catabolism of plant hormones plays significant roles in plant growth and development (Zhang et al. 2013; Koo et al. 2014; Kramer and Ackelsberg 2015).

According to previous reports, either reduced cytokinin levels or deficient cytokinin perception can result in slower growth rates and reduced plant stature in the transformants and mutants of *Arabidopsis* (Werner et al. 2003; Higuchi et al. 2004; Nishimura et al. 2004; Riefler et al. 2006), indicating that controlling the bioactive cytokinin status is essential for SAM maintenance and function. In this study, we provide several lines of evidence supporting that *OscZOG1* is a critical player in maintaining the activities of SAM and RAM in rice. Firstly, in situ hybridization data suggest that *OscZOG1* expression is highly targeted to cells in rapidly growing regions, including shoot and root meristematic tissues. Secondly, genetic modulation of the *OscZOG1* expression can act as a fine-tuning of regulating the shoot development, especially for the yield contributing factors such as tiller number, panicle branches and total spikelet number per panicle. Thirdly, in accordance with the role of cytokinin in regulating lateral root organogenesis (Laplaze et al. 2007; Hwang et al. 2012), our results indicate that modulating the expression levels of *OscZOG1* evidently affects the initiation and growth of lateral roots in rice. As one of the central endogenous signaling

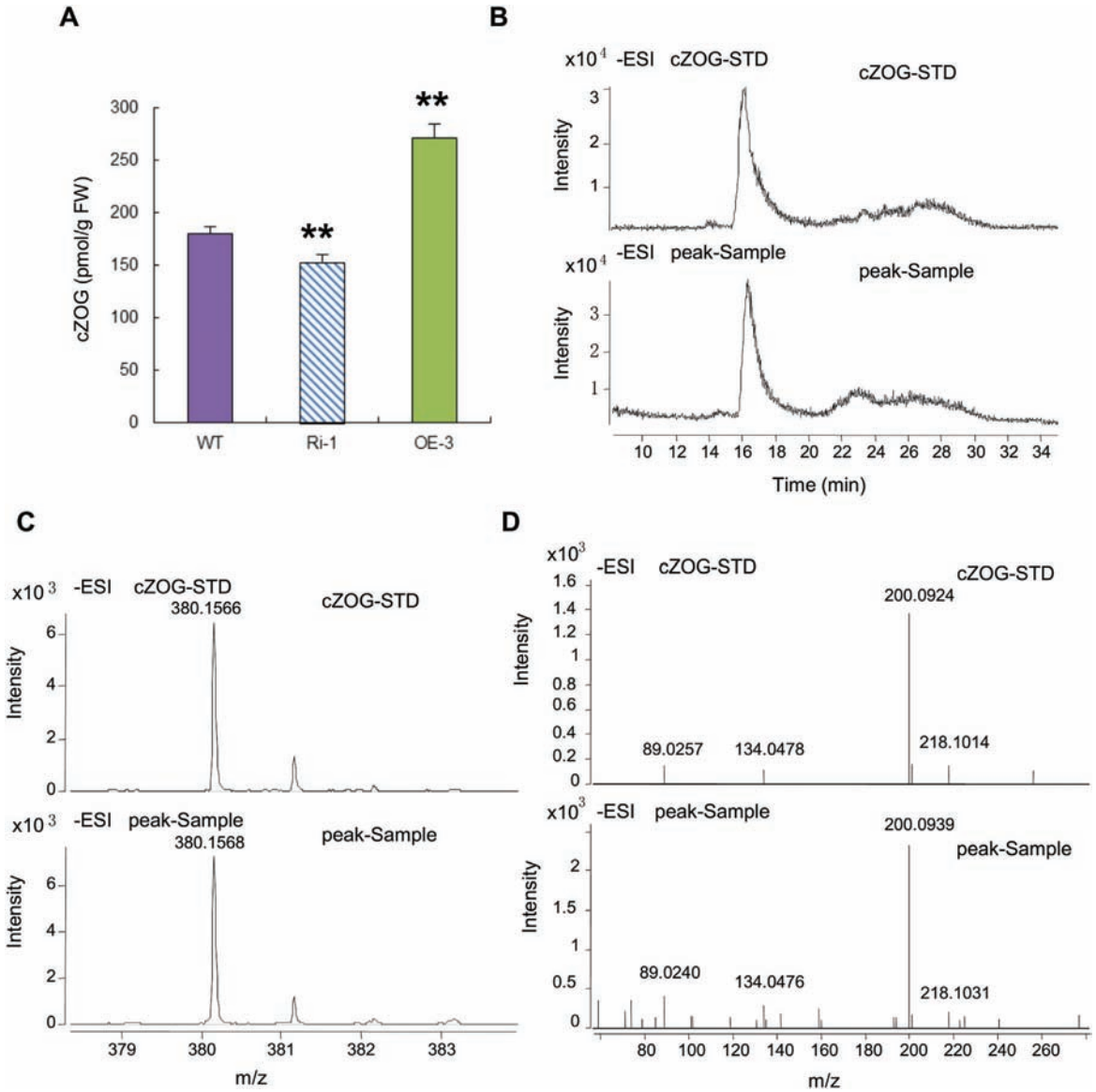

Figure 10. Over-expressing *OscZOG1* leads to cZOG accumulation in the transgenic rice plants
(**A**) Contents of cZOG in shoots of WT, the RNAi and overexpression transgenic lines analyzed by HPLC-MS/MS. Plants were grown in greenhouse and shoots were harvested at 35 DAG for the measurements of endogenous cytokinins. Values are means (+/− SD) of three plants. Statistical analyses were performed ($^{**}P < 0.01$, Student's *t*-test). Error bars indicate *SD*. (**B**–**D**) HPLC-MS/MS analysis of cZOG in shoot samples. (**B**) Chromatographical profiles of cZOG in plant extracts (peak-sample) compared with the standard of cZOG (cZOG-STD). (**C**) and (**D**) The mass spectra (**C**) and MS/MS spectra (**D**) of the peaks corresponding to peak-sample and the standard of cZOG.

molecules, the plant hormone cytokinins tightly control the dividing activities of founder cells during the early phases of lateral root organogenesis (Laplaze et al. 2007). Our findings reveal a critical role of *OscZOG1* in modulating the cytokinin response, which ensures continuous lateral root initiation (LRI) and proper development of lateral root primordia (LRP).

Very few genes specific to cytokinin conjugation have been isolated in positively regulating the formation of yield-contributing agronomic traits. Thus, *OscZOG1*, encoding a putative zeatin metabolic enzyme, is useful for the common genetic control of cytokinin glucosylation levels, which will result in a cumulative increase in yield due to the integrative positive effects on meristematic activity in both vegetative and reproductive developmental stages. It is accepted that *trans*-zeatin is the most active and ubiquitous cytokinin and cytokinin *O*-glucosides have been assumed to represent reversibly inactivated storage forms (Mok et al. 2000b; Mok and Mok 2001). However, recent studies have provided several lines of evidence suggesting that *cis*-zeatin (cZ) could act as an active cytokinin in maize (Martin et al. 2001b; Veach et al. 2003) and rice (Kudo et al. 2012). The genetic modulation of *OscZOG1* expression reported here exploits the conjugation

properties of *cis*-zeatin to regulate the bioactive levels of cytokinins critical for the activity of shoot and inflorescence meristems, indicating that *OscZOG1*-mediated glucosylation of *cis*-zeatin has a regulatory function. More importantly, *O*-glucosylzeatin is found in all plant species examined (Mok et al. 2000a, 2000b; Mok and Mok 2001; Bajguz and Piotrowska 2009). Thus, our findings have provided the opportunity for fully characterizing the role of *OscZOG1* as a critical component in balancing cytokinin homeostais during the formation of yield-contributing traits and as points for the artificial manipulation of cytokinin-mediated growth control. Since it has long been recognized that grain development is related to elevated zeatin in cereals (Cheikh and Jones 1994; Dietrich et al. 1995; Ashikari et al. 2005; Wilkinson et al. 2012), the dissection of molecular mechanisms of cytokinin-glucosylation that contributes to the formation of rice yield traits is important for developing high-yielding rice varieties. Our data also inform the potential advantage of modulating *OscZOG1* expression as a fine-tuning switch to improve multiple favorable agronomic traits in one genetic background, which could circumvent some of the pitfalls so far encountered in breeding for increases in the complex traits of yield.

MATERIALS AND METHODS

Plant material and growth conditions

The rice (*Oryza sativa japonica*) variety Zhonghua 11 was used as wild-type. The wild-type and transgenic plants were grown in an isolated rice paddy field. For the seedling examination, the seedlings were cultured in Yoshida's culture solution (Huang et al. 2009) under a 12/12-h day/night condition at 26 °C for 2 weeks. For the experiments of in situ hybridization and cytokinin quantification, plants were grown in soil in greenhouse supplied with a 12/12-h day/night regime at 28 °C.

Generation of OscZOG1-overexpression and -RNAi transgenic plants

The full-length CDS of *OscZOG1* (Os04g20330), amplified by RT-PCR with the primers of OE-F (5′-GTAAGCTTATGCCCAGCGATGGCAGCTT-3′) and OE-R (5′-GCTCTAGATCAGCATTTCATGAGCATAA-3′), were cloned into the pHB binary vector with the *HindIII* and *XbaI* sites to generate the overexpression (OE) constructs. For generating *OscZOG1*-RNAi constructs, a 496 bp fragment, starting at 34 bp upstream and ending at 461 bp downstream of the ATG codon of *OscZOG1* was amplified separately with sense primers RiF1 (5′- CAGCTAGCATCGATGGAGAACCTCGAAGTCAA-3′) and RiR1 (5′-GCAGATCTACTAGTCCGCAGGAGACGAAGATG-3′) and antisense primers RiF2 (5′-CACCCGGGGGAGAACCTCGAAGTCAA-3′) and RiR2 (5′-CAGGATCCCCGCAGGAGACGAAGATG-3′). The amplified sense and antisense fragments were subcloned into the rice RNAi binary vector pTCK303 (Wang et al. 2004) with the restriction sites of *SpeI*/*ClaI* (sense) and SmaI/BamHI (antisense). The agrobacterium-mediated transformation of Zhonghua 11 was performed as described (Toki et al. 2006).

Real-time RT-PCR analysis

Total RNA was isolated from samples frozen in liquid nitrogen using RNAiso Plus Reagent (Takara) according to the manufacturer's instructions. DNA contaminated in total RNA samples was digested with RNase-free DNase (Takara). Following the treatment with DNase, ReverTra Ace reverse transcriptase (TOYOBO) was used to synthesize complementary DNA (2 μg total RNA as template) using an oligo $(dT)_{18}$ (Takara) as a primer. The comparative threshold cycle (Ct) method was used for determining relative transcript levels (iQ5 admin, Bio-Rad) using *OsACTIN* (LOC_Os03g50885) as an internal control. Quantitative real-time PCR was performed with SYBR Premix Ex TaqII (Takara) using an MyiQ5 single color Real-Time PCR Detection System (Bio-Rad).

The primers of qcZOGF (5′-CCGTTCGGGTTTGACATCG-3′) and qcZOGR (5′-GTTCTTGGCACGCATCCTCTC-3′) were used for the amplification of *OscZOG1*. *OsACTIN* was amplified using the primers of qACTF (5′-TGGTCGTACCACAGGTATTGTGTT-3′) and qACTR (5′-AAGGTCGAGACGAAGGATAGCAT-3′).

Histological analysis and in situ hybridization

Plant samples were fixed in FAA solution (50% ethanol, 5% acetic acid, 3.7% formaldehyde) at 4 °C overnight, followed by a series of dehydration and infiltration, and embedded in paraffin (Paraplast Plus, Sigma-Aldrich). The tissue samples were sliced into 9 μm sections with a microtome (Leica RM2235), affixed to microscope slides. Sections were observed and photographed under bright field by a microscope (Olympus BX51) or used for in situ hybridization.

To prepare the probes of *OscZOG1* used for in situ hybridization, a 427 bp fragment was amplified with the primers InsF (5′-GGCAGGCCGTTCATTTGG-3′) and InsR (5′-TGTCCGCCTTCGCCGTAA-3′) using *OscZOG1* cDNA as a template. The amplified fragments were introduced into pMD19-T vector (Takara) and sequenced. The resulting fragment was then cloned into pBluscript SK vector with the restriction sites *PstI* and *BamHI* and lineared by *EcoRI* (anti-sense) or *BamHI* (sense) as a template for the generations of digoxigenin-labeled RNA probes. The sense or antisense RNA probes labeled with digoxigenin (Roche) were produced by T7 and T3 transcriptase, respectively. RNA in situ hybridization was performed as described (Long and Barton 1998).

Quantification of endogenous cytokinins

For extraction and purification of cytokinins, the procedures were followed as described (Chen et al. 2010). The shoots (about 1 g fresh weight) were harvested from 35-d-old rice plants grown in greenhouse. Tissues were homogenized to powders with a pestle in a ceramic mortar in liquid nitrogen. The resulting powders were extracted with 5 mL cold (−20 °C) extraction mixture of methanol/water/formic acid (15:4:1,v/v/v) in 50 mL polypropylene centrifuge tubes at −20 °C overnight. The resulting extracts were centrifuged at 10 000*g* at 4 °C for 30 min. With the supernatant being decanted, the remaining residues were re-extracted for 1 h in additional 5 mL extraction mixture at −20 °C and centrifuged as above. The resulting supernatants were pooled and then passed through a Sep-Pak Plus C18 cartridge (Waters) to remove lipids and plant pigments. The residue was dissolved in 5 mL 1 M formic acid and applied to an Oasis MCX column (Waters) after the methanol in extracts was removed *in vacuo*. The column was washed with 5 mL 1 M formic acid, 5 mL methanol, and then 5 mL 0.35 M NH_4OH when samples were loaded. The cytokinin bases and their corresponding glucosides/ribosides were eluted with 5 mL of 0.35 M NH_4OH in 60%

methanol. The eluate was evaporated *in vacuo*. The resulting residues were re-suspended in 50 μL of 0.1% acetic acid-water and passed through a micro-filter.

Endogenous cytokinins in the filtrate were detected and quantified by HPLC-MS/MS system (Agilent 6520 Accurate-Mass Q-TOF) equipped with a C18 column (Zorbax SDB-C18, 4.6 × 50 mm, 1.8 μm, Agilent). Two deuterium-labeled cytokinins ([$^2H_5$] *trans*-zeatin, [$^2H_5$] *trans*-zeatin-*O*-glucoside, OlChemim Ltd., Olomouc, Czech Republic), each at 50 ng per sample, were used as internal standards. Because deuterated standards of *cis*-zeatin and derivatives are not available, the levels of these compounds were calculated based on the recovery of deuterated standards of the corresponding trans compounds as described (Veach et al. 2003). Linear gradients of methanol (B) in 0.1% (v/v) formic acid in water (A) were used according to the following profile: 0 min, 92%A + 8% B; 15 min, 85%A + 15%B; 20 min, 10%A + 90% B; 23 min, 10%A + 90% B; 25 min, 92%A + 8%B. The flow rate was 0.2 mL min^{-1}. Data were processed by G3335AA MassHunter Qualitative Analysis Software (Agilent).

Subcellular localization analysis

For generating *OscZOG1-GFP* constructs, the full-length CDS of *OscZOG1* was amplified by RT-PCR with the primers of GFPF (5′-AGCTCGAGTATGCCCAGCGATGGCAGC-3′) and GFPR (5′- CACTAGTAGGCATTTCATGAGCATAAT-3′). The resulting fragment was introduced into 35S-GFP-JFH1 vector(Hong et al. 1999) with the restriction sites *XhoI* and *SpeI* to generate a C-terminal GFP fusion construct. The *OscZOG1-GFP* constructs were transferred into *Agrobacterium tumefaciens* strain GV3101. Transgenic plants were generated by a floral dip method and screened on solid plates containing 50 mg L^{-1} kanamycin. Leaves of 24-d-old transgenic plants were used for protoplast extractions as reported (Yoo et al. 2007).

Confocal microscopy

GFP images were visualized by a LSM510 laser scanning confocal microscopy (Zeiss, Jena, Germany) with argon laser excitation at 488 nm and a 505–550-nm emission filter set for GFP fluorescence.

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AUTHOR CONTRIBUTIONS

F.Q.G. and X.L.S. designed the experiment and F.Q.G. supervised the study. X.L.S. performed most of the research. F.Q.G. and X.L.S. drafted and revised the manuscript. X.L.S., H.T. and R.R.X. carried out in situ hybridization experiments, R.R.X. and Q.L.W. performed some expression analyses.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Effects of overexpression or interference with *OscZOG1* expression on flag leaf senescence in rice

Figure S2. Phylogenetic relations of OscZOG1 and other reported zeatin-*O*-glucosyltransferases

Figure S3. Subcellular localization patterns of OscZOG1-GFP in *Arabidopsis* protoplasts isolated from leaves of the *35S:: OscZOG1-GFP* transgenic plants

Figure S4. Effects of overexpression or interference with *OscZOG1* expression on accumulation of cytokinins in the transgenic rice plants